

The testicular transcript of the angiotensin I-converting enzyme encodes for the ancestral, non-duplicated form of the enzyme

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The endothelial angiotensin I-converting enzyme (ACE) is organized in two large homologous domains, each bearing a putative active site. However, only one of these sites is probably involved in catalyzing the conversion of angiotensin I into angiotensin II. The testicular form of ACE is equally active, encoded by the same gene, but translated from a shorter mRNA. Molecular cloning of the human testicular ACE cDNA indicates that the mRNA codes for 732 residues (vs 1306 in endothelium). The testicular transcript corresponds to the 3' half of the endothelial transcript and encodes one of the two homologous domains of endothelial ACE, preceded by a short specific sequence. This 5' specific sequence contains 228 nucleotides and encodes 67 amino acids, including the putative signal peptide followed by a serine/threonine-enriched region, presumably glycosylated. The testicular transcript corresponds to the ancestral, non-duplicated form of the ACE gene. Since the carboxyl-terminal domain of the endothelial ACE is expressed in the testicular enzyme, it is likely that it bears the active site in both forms.

Molecular cloning; Metalloproteinase; Membrane protein; Differential splicing; (Germ cell)

1. INTRODUCTION

Two immunologically and enzymatically related forms of angiotensin-I converting enzyme [peptidyl-dipeptide hydrolase, EC 3.4.15.1 (ACE)] are present in mammals. Whereas in vascular endothelial and absorptive epithelial cells ACE is in the large, single-chain, membrane-bound form with a molecular mass of around 170 kDa, in male germ cells it is synthesized as a shorter polypeptide and of approx. 100 kDa [1–4]. Molecular cloning of human endothelial ACE cDNA allowed us to establish that the testicular and endothelial enzymes are encoded by two structurally related mRNAs of different length, 3.0 and 4.3 kb, respectively, both transcribed from a single gene [5]. We undertook the cloning of human testicular ACE cDNA to elucidate the structural relationship between both forms of ACE. Further interest in

this study arose from the observation in endothelial ACE of internal homology between two large peptide domains, each bearing a putative active site. Our results show that only one of the two domains is expressed in the testicular enzyme, although this enzyme displays enzymatic properties apparently identical to those of endothelial ACE.

2. MATERIALS AND METHODS

2.1. Preparation and screening of cDNA libraries

A human testis cDNA library, primed with oligo(dT) and constructed in bacteriophage λ gt11 (Clontech, Palo Alto, CA), was screened with the 32 P-labeled λ HEC1922 cDNA clone previously isolated in this laboratory and corresponding to the nucleotide sequence of the human endothelial ACE mRNA from position 691 to 4024 [5]. λ HEC1922 cDNA was labeled using the random primer method [6]. Procedures for screening the λ phage cDNA library, hybridization with the labeled probe and washing under stringent conditions were as described in [5] according to Benton and Davis [7]. Clones corresponding to the 3' part of the testicular cDNA were obtained (fig.1). A synthetic oligomer (TH16), corresponding to the nucleotide se-

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3. RESULTS

By screening 2×10^6 clones of the oligo-d(T)-primed testis library with endothelial clone λ HEC1922, which gave strong hybridization with testicular poly(A) RNA by Northern blot analysis [5], 17 putative clones were obtained. Two of the clones, λ ht10 and λ ht16, bearing 2.2 and 2.3 kb inserts respectively, were sequenced. They contain a sequence identical to the 3' part of endothelial ACE cDNA, but extend more in the 3' untranslated region. Clone λ ht10 contains the longest 3' untranslated region with a polyadenylation signal ATAAA followed by a poly(A) tail and spans nucleotide positions 260–2477 of ACE testicular cDNA (fig.1). This unusual polyadenylation sequence has been already found in other mammalian genes [10]. The nucleotide sequence of this clone matches completely with the 3' sequenced part of the endothelial ACE mRNA [5]. Clone λ ht16 corresponds to positions 50–2313 of

testicular ACE mRNA. Upstream of nucleotide position 228, the sequence of the cDNA displays no similarity to that of endothelial ACE cDNA (figs 2,3). After a second screening of the cDNA library with oligomer TH16, corresponding to the 5' part of clone λ ht16 (see above), clone λ ht341 was obtained. This clone, 2.2 kb in length, has a sequence identical to that of λ ht16, including the 5' testicular specific region, and extends 19 nucleotides upstream to the 5' extremity of λ ht16. No putative initiation codon ATG was found in the 5' sequence of these clones.

The cDNA corresponding to the 5'-end of the mRNA was obtained from the testis cDNA library primed with oligomer ATH17 (see section 2). Eight positive clones were obtained by screening this library with the oligomer TH16. One of the clones, λ ht22, 244 bp in length, was sequenced. It displays the 5' part of the testicular ACE mRNA sequence with the putative initiator codon preceded by a 28-base 5' untranslated region (fig.1). The consen-

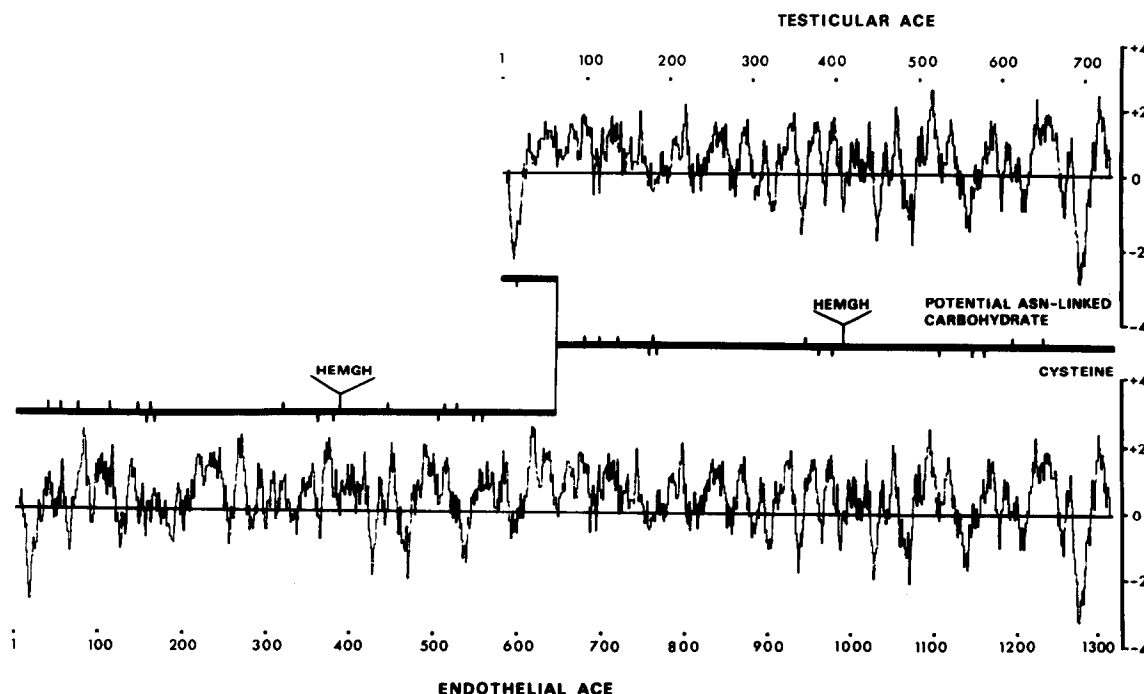


Fig.3. Schematic representation of testicular and endothelial ACE. (Middle) Diagram showing cysteine positions, potential asparagine-linked glycosylation sites, and positions of putative residues of the active site of the two enzymes (HEMGH). Beyond the point of divergence, the testicular enzyme is shown on the upper line and the endothelial enzyme on the lower line. (Top, bottom) Hydropathy plots of the predicted testicular (top) and endothelial [5] (bottom) amino acid sequences by the method of Kyte and Doolittle with a window size of 10 residues [18]. Negative values indicate increasing hydrophobicity. Amino acid numbering presented above and below the hydropathy plots.

sus sequence for initiation of translation is found from position 26 to 32 [11].

The composite nucleotide sequence of the testicular ACE cDNA obtained from the four overlapping clones comprises 2477 nucleotides (fig.2). The open reading frame from the first ATG codon until the stop codon TGA encodes 732 amino acids. The calculated molecular mass of the testicular ACE precursor is 83.2 kDa whereas its value was 149.5 kDa for the endothelial ACE [5].

In another clone, isolated from the oligo(dT)-primed cDNA library, a deletion of 97 bases is observed between nucleotide positions 130 and 228 of testicular ACE cDNA. The sequence of the 5' edge of the deletion is GT, preceded by AG, and has been recognized probably as an internal acceptor site. The 3' edge of the deletion corresponds to the junction between the specific testicular part of the mRNA and the common sequence with the endothelial mRNA. This aberrant splicing inside the 3' testicular exon generates another reading frame that terminates shortly thereafter by a stop codon.

4. DISCUSSION

Cloning of the cDNA for testicular ACE and alignment with cDNA for the endothelial enzyme indicate that the mRNAs of both enzymes share in their 3' region a large identical sequence that comprises the last 1997 nucleotides of the coding sequence. However, the 5' part of the transcript differs between the two enzymes. In testicular cDNA, the 5' part of the sequence down to the point of convergence with the sequence of the endothelial enzyme contains the initiator ATG codon followed by the sequence corresponding to the signal peptide (see below) and comprises 228 nucleotides. This sequence is not found in the 1943-nucleotide-long 5' specific part of the endothelial enzyme cDNA [5]. As a unique gene encodes both forms of ACE in man [5], the testicular and endothelial mRNAs may be generated by differential splicing of a common primary transcript. Alternatively, they could be transcribed from two different transcription start sites under the control of separate promoters [12]. This latter hypothesis is supported by our observation in genomic clones corresponding to the 5'-end of testicular cDNA of a TATA-related sequence situated 19 bases

upstream of the 5' extremity of testicular mRNA (Hubert, C. et al., in preparation). The presence of alternative promoters with different tissue specificity is also in agreement with probable differences in hormonal regulation. The testicular enzyme is indeed expressed in the mature forms of germ cells [13–16] and is probably under the control of androgens [17]. The demonstration of a functional testicular promoter requires further studies, such as transfection experiments.

The protein sequence deduced from the testicular cDNA comprises 732 amino acids. The first 67 amino acids are translated by the specific testicular portion of the mRNA and therefore are not found in the endothelial enzyme. The last 665 residues are identical to the carboxyl-terminal part of the endothelial ACE (fig.3).

A computer search revealed no clear significant homology of the specific testicular part of the sequence with any other protein found in the NBRF bank (release 19.0). No homology was found by alignment with the amino- or carboxyl-terminal peptide sequences reported for rabbit testicular ACE [19]. The sequence begins with a peptide sequence bearing the characteristics of a signal peptide [20]. The probable cleavage site of the signal peptide in the testicular enzyme, as predicted by the program of Von Heijne [21], is between amino acid positions 21 and 22. In endothelial ACE, the signal peptide is cleaved off during maturation and the enzyme becomes anchored to the plasma membrane by the 17-amino-acid transmembrane domain situated near its carboxyl-terminal extremity [5]. The testicular enzyme, that is also, at least in part, in a membrane-bound form [15,22], possesses this transmembrane segment (fig.3). Like endothelial ACE, it may be anchored by this carboxyl-terminal region after cleavage of the signal peptide, although this should be confirmed by sequencing of the amino- and carboxyl-terminal extremities of the mature enzyme.

The peptide sequence that follows the putative signal peptide is hydrophilic (fig.3) and has an unusually high content of threonine and serine: the sequence situated between Ser 28 and 65 contains 47% threonine and serine. Similar serine- and threonine-enriched sequences are found in membrane-bound proteins, situated externally near the region of membrane insertion, e.g. in LDL receptor, aminopeptidase N, sucrase-

isomaltase complex, and interleukin II receptor, or close to the amino-terminal floating extremity in glycophorin A [23–28]. They are potential sites of clustered *O*-glycosylation. Evidence for clustered *O*-glycosylation has not been provided for the testicular ACE, but it is known that rabbit testicular ACE, in contrast to the pulmonary enzyme, has a large content of *N*-acetylgalactosamine [1], a typical constituent of *O*-branched glycans [29]. This may reflect clustered *O*-glycosylation in the specific amino-terminal part of the testicular ACE sequence.

The endothelial ACE molecule is organized in two large homologous domains separated by a short non-homologous region [5]. Identity with the testicular enzyme begins in the interdomain region and testicular ACE comprises only the carboxyl-terminal domain (fig.3). As discussed for the endothelial enzyme, this domain bears sequences identical to those located around critical residues of the other metallopeptidases, thermolysin, neutral endopeptidase and collagenase [5]. Therefore, His 414, 418 and Glu 442 are probably the amino acids coordinating the zinc atom in the testicular enzyme, with Glu 415 and His 457 being involved in catalysis.

In the endothelial enzyme each of the two homologous domains bears a putative active site [5]. The testicular form of the enzyme, despite the presence of only one active site, apparently displays enzymatic properties identical to those of the endothelial enzyme, including the K_m and turnover number for angiotensin I, as well as chloride dependency for hydrolysis [1]. Its function in germ cells remains unknown and may not be related to angiotensin II formation. To date, there is no evidence of the testicular enzyme acting as a dimer. A lower molecular mass was indeed observed for the testicular vs pulmonary form of ACE by submitting the native rabbit enzymes to density gradient centrifugation [1]. The elucidation of the structure of the testicular enzyme suggests that despite the repetitive structure of endothelial ACE, only one of the two homologous domains expresses the dipeptidyl peptidase activity. This is also in agreement with the observation of a single zinc atom bound per molecule of endothelial ACE, and of a unique binding site for competitive inhibitors (see [5]). Comparison with the testicular enzyme suggests that the carboxyl-terminal part of

the molecule bears the active site in endothelial ACE.

The results of this study, indicating that the testicular ACE transcript corresponds to only one of the two homologous regions of the endothelial transcript, are in agreement with the hypothesis that endothelial ACE resulted from gene duplication. Testicular ACE mRNA indeed corresponds to transcription of the putative ancestral form of the gene, before the occurrence of duplication. This ancestral gene was apparently able to encode a fully active enzyme. The consequences for ACE activity, if any, of the appearance of the duplicated endothelial form remain to be established.

REFERENCES

- [1] El Dorry, H., Bull, H.G., Iwata, K., Thornberry, N.A., Cordes, E.H. and Soffer, R.L. (1982) *J. Biol. Chem.* 257, 14128–14133.
- [2] El Dorry, H.A., Pickett, C.B., Mac Gregor, J.S. and Soffer, R.L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 4295–4297.
- [3] Lanzillo, J.J., Stevens, J., Dasarathy, Y., Yotsumoto, H. and Fanburg, B.L. (1985) *J. Biol. Chem.* 260, 14938–14944.
- [4] Erdős, E.G. and Skidgel, R.A. (1987) *Lab. Invest.* 56, 345–348.
- [5] Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, J. and Corvol, P. (1988) *Proc. Natl. Acad. Sci. USA* 85, 9386–9390.
- [6] Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6–13.
- [7] Benton, W.D. and Davis, R.W. (1978) *Science* 196, 180–182.
- [8] Chirgwin, J.M., Przybyla, A.E., Mac Donald, R.J. and Rutter, W.J. (1979) *Biochemistry* 18, 5294–5299.
- [9] Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C. and Kunkel, L.M. (1987) *Cell* 50, 509–517.
- [10] Zakut, R., Shani, M., Givol, D., Neuman, S., Yaffe, D. and Nudel, U. (1982) *Nature* 298, 857–859.
- [11] Kozak, M. (1987) *Nucleic Acids Res.* 15, 8125–8148.
- [12] Breitbart, R.E., Andreadis, A. and Nadal-Ginard, B. (1987) *Annu. Rev. Biochem.* 56, 467–495.
- [13] Cushman, D.W. and Cheung, H.S. (1971) *Biochim. Biophys. Acta* 250, 261–265.
- [14] Vanha-Perttula, T., Mather, J.P., Bardin, C.W., Moss, S.B. and Bellve, A.R. (1985) *Biol. Reprod.* 33, 870–877.
- [15] Berg, T., Sulner, J., Lai, C.Y. and Soffer, R.L. (1986) *J. Histochem. Cytochem.* 34, 753–760.
- [16] Saint-Andre, J.P., Alhenc-Gelas, F., Rohmer, V., Chretien, M.F., Bigorgne, J.C. and Corvol, P. (1988) *Hum. Pathol.* 19, 208–213.
- [17] Velletri, P.A., Aquilano, D.R., Bruckwick, E., Tsai-Morris, C.H., Dufau, M.L. and Lovenberg, W. (1985) *Endocrinology* 116, 2516–2522.

- [18] Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* 157, 105–132.
- [19] Iwata, K., Lai, C.H., El-Dorry, H. and Soffer, R.L. (1982) *Biochem. Biophys. Res. Commun.* 107, 1097–1103.
- [20] Perlman, D. and Halvorson, H.O. (1983) *J. Mol. Biol.* 167, 391–409.
- [21] Von Heijne, G. (1983) *Eur. J. Biochem.* 133, 17–21.
- [22] Stritmatter, S.M. and Snyder, S.H. (1984) *Endocrinology* 115, 2332–2341.
- [23] Cummings, R.D., Kornfeld, S., Schneider, W., Hobgood, K., Tolleshaug, H., Brown, M.S. and Goldstein, J.L. (1983) *J. Biol. Chem.* 258, 15261–15273.
- [24] Olsen, J., Cowell, G.M., Konigshofer, E., Danielsen, E.M., Moller, J., Laustsen, L., Hansen, O.C., Welinder, K.G., Engberg, J., Hunziker, W., Spiess, M., Sjöström, H. and Noren, O. (1988) *FEBS Lett.* 238, 307–314.
- [25] Hunziker, W., Spiess, M., Semenza, G. and Lodish, H. (1986) *Cell* 46, 227–234.
- [26] Leonard, W.J., Depper, J., Crabtree, G.R., Rudikoff, S., Pumphrey, J., Robb, R.J., Kronke, M., Svetlik, P.B., Pfeffer, N.J., Waldmann, T.A. and Greene, W.C. (1984) *Nature* 311, 626–631.
- [27] Nikaido, T., Shimizu, A., Ishida, N., Sabe, H., Teshigawara, K., Maeda, M., Uchiyama, T., Yodoi, J. and Honjo, T. (1984) *Nature* 311, 631–635.
- [28] Tomita, M. and Marchesi, V.T. (1975) *Proc. Natl. Acad. Sci. USA* 72, 2964–2968.
- [29] Montreuil, J. (1982) in: *Comprehensive Biochemistry* (Neuenberger, A. and Van Deenen, L. eds) vol.19B (II), pp.1–188, Elsevier, Amsterdam.